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Flow Cytometry Analyses of Adipose Tissue Macrophages

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Abstract

Within adipose tissue, multiple leukocyte interactions contribute to metabolic homeostasis in health as well as to the pathogenesis of insulin resistance with obesity. Adipose tissue macrophages (ATMs) are the predominant leukocyte population in fat and contribute to obesity-induced inflammation. Characterization of ATMs and other leukocytes in the stromal vascular fraction from fat has benefited from the use of flow cytometry and flow-assisted cell sorting techniques. These methods permit the immunophenotyping, quantification, and purification of these unique cell populations from multiple adipose tissue depots in rodents and humans. Proper isolation, quantification, and characterization of ATM phenotypes are critical for understanding their role in adipose tissue function and obesity-induced metabolic diseases. Here, we present the flow cytometry protocols for phenotyping ATMs in lean and obese mice employed by our laboratory.

1. INTRODUCTION

Obesity induces a low-grade inflammatory state that contributes to insulin resistance, diabetes, and metabolic syndrome (Glass & Olefsky, 2012; Gregor & Hotamisligil, 2011; Lumeng & Saltiel, 2011; Xu et al., 2003). Obesity-induced inflammation is characterized by chronic elevations in circulating inflammatory cytokines, adipokines, and monocytes (Gregor & Hotamisligil, 2011). At the tissue level, inflammatory pathways are induced in visceral adipose tissue due in part to dynamic quantitative and phenotypic changes in adipose tissue leukocytes, which include macrophages, neutrophils, mast cells, T cells, and eosinophils (Liu et al., 2009; Nishimura et al., 2009; Strissel et al., 2010; Talukdar et al., 2012; Wu et al., 2011). Among these, adipose tissue macrophages (ATMs) are the predominant leukocyte population in fat (Nishimura et al., 2009; Wentworth et al., 2010). In both mouse models and human subjects, obesity leads to increased ATM accumulation in visceral adipose depots (Harman-Boehm et al., 2007; Weisberg et al., 2003; Xu et al., 2003). In mouse models, ATM content can increase from ~10% to 15% of nonadipocyte cells in fat in lean mice to ~50% of cells in obese mice (Weisberg et al., 2003; Xu et al., 2003). ATM content positively correlates with the metabolic derangements associated with obesity in rodent and humans (Kanda et al., 2006; Wentworth et al., 2010; Xu et al., 2003).

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Obesity is also associated with qualitative changes in the phenotype and function of ATMs. In lean mice, resident ATMs are distributed between adipocytes in healthy adipose tissue and express anti-inflammatory markers typical of “alternatively activated” or M2-polarized macrophages (e.g., arginase 1, CD301/Mgl1, and CD206) (Lumeng, Bodzin, & Saltiel, 2007). Dietary obesity triggers the accumulation of ATMs into “crown-like structures” around dead adipocytes (Cinti et al., 2005; Strissel et al., 2007). These infiltrating ATMs express the dendritic cell marker CD11c and genes typical of “classically activated” or M1-polarized macrophages (Lumeng, Bodzin, & Saltiel, 2007). Recruited $CD11c^+$ ATMs secrete proinflammatory cytokines such as TNF-α and IL-6 and generate reactive oxygen species via inducible nitric oxide synthase (NOS2) (Lumeng, DelProposto, Westcott, & Saltiel, 2008; Lumeng, Deyoung, Bodzin, & Saltiel, 2007). Collectively, these and other observations have led to the paradigm that ATMs undergo a “phenotypic switch” from an anti-inflammatory M2 state to a proinflammatory M1 state (Lumeng, Bodzin, & Saltiel, 2007). While this is an oversimplification of a complex regulatory system, evidence from knockout mice support the general model M1/M2 balance in macrophages can play a pivotal role in the development of adipose tissue inflammation in obesity (Chawla, Nguyen, & Goh, 2011; Lumeng & Saltiel, 2011).

The limited number and complex heterogeneity of stromal vascular cells (SVCs) isolated from fat depots poses a challenge on the types of analyses that can be applied to directly study ATMs. *In vitro* assays using bone marrow-derived macrophages or macrophage cell lines are limited in that they may not recapitulate the ATM microenvironment. The use of flow cytometry has emerged as the preferred method to interrogate ATM content and heterogeneity in mouse models. When done properly flow cytometry allows investigators to simultaneously examine both general cell properties (e.g., relative size and granularity) and expression of extracellular and intracellular proteins on individual cells isolated from fat. In concert with purification schemes such as immunomagnetic cell enrichment and flow-assisted cell sorting (FACS), flow cytometry becomes an invaluable tool for studying ATMs and other leukocytes in adipose tissue. Technical approaches vary from laboratory to laboratory making it somewhat of a challenge to interpret data across studies. Much of this may stem from the use of collagenase digestion protocols originally developed for adipocyte isolation that may not adequately capture all leukocytes for downstream analysis (Rodbell, 1964). In this chapter, we provide the protocol used by our group optimized for detection and purification of ATM subsets by flow cytometry. We provide several practical considerations for optimizing cell yield, for selecting proper reagents and flow cytometry controls, and for gating SVCs to characterize distinct ATM subsets.

2. MATERIALS

2.1. Isolation and preparation of SVCs from mice

1. C57BL/6J mice (The Jackson Laboratory, Bar harbor, ME; stock #00064)
2. Sterile or ethanol-cleaned surgical instruments
3. 70% Ethanol
4. 10 cc Luer-Lok syringes with needles (25G×1″)

5. 1× Phosphate-buffered saline (PBS)
6. Digestion buffer: Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} supplemented with 0.5% bovine serum albumin (BSA)
7. 10× Collagenase solution (10 mg/ml in digestion buffer). Type II collagenase (Sigma-Aldrich; Catalog #C6885). Solution should be prepared freshly for optimal results and can be filter-sterilized (0.22 μm)
8. 100 μm Nylon cell strainers (BD Falcon; Catalog #352360)
9. 1× RBC lysis buffer: 155 m*M* NH_4Cl, 10 m*M* $KHCO_3$, 0.1 *M* EDTA; sterile filtered through 0.22-μm filter and store in aliquots at 4 °C
10. 0.5 *M* EDTA
11. FACS buffer: PBS with 1 m*M* EDTA, 25 m*M* HEPES, and 1% heat-inactivated fetal bovine serum (FBS)
12. Trypan blue solution, 0.4% (Invitrogen; Catalog #15250)
13. Refrigerated clinical centrifuge with swing-bucket rotors and adapters for 15- and 50-ml conical tubes
14. Incubator set to 37 °C
15. Test tube rocker/mixer
16. Hemocytometer for counting cells

2.2. Staining SVCs for cell surface markers to identify ATMs

1. 5-ml Polystyrene round-bottom test tubes for flow cytometry (12×75 mm)
2. Fluorochrome-labeled anti-mouse monoclonal antibodies against indicated cell surface molecules (Table 16.1)
3. Viability dye: DAPI or Live/Dead Fixable Dead Cell Kit (Life Technologies; Catalog #L34955)
4. 16% Paraformaldehyde (PFA) (EM grade)

2.3. Flow cytometry analysis

1. Flow cytometer (e.g., FACSCanto II; BD Biosciences) equipped with three lasers (405-nm violet laser, 488-nm blue laser, and 640-nm red laser) and detectors for the indicated fluorochromes
2. SVCs stained with each individual fluorochrome-labeled antibody (single stained (SS) controls) and fluorescence minus one (FMO) controls (Table 16.2)

2.4. FACS procedure to purify ATM populations

1. Cell sorter (e.g., FACSAria II; BD Biosciences) equipped with three lasers (405-nm violet laser, 488-nm blue laser, and 640-nm red laser) and detectors for the indicated fluorochromes

2. FACS buffer: PBS with 1 m*M* EDTA, 25 m*M* HEPES, and 1% heat-inactivated FBS

2.5. Using magnetic beads and positive selection to enrich for CD11b$^+$ SVCs

1. 5-ml Polystyrene round-bottom test tubes for flow cytometry (12×75 mm)
2. MACS buffer: PBS (without Ca^{2+} and Mg^{2+}) supplemented with 0.5% BSA
3. CD11b microbeads (Miltenyi Biotec; Catalog #130-049-601)
4. MACS MS cell separation columns with corresponding MACS separator magnet (Miltenyi Biotec, Bergisch Gladbach, Germany)

2.6. Data analysis

1. Flow cytometry analysis software (e.g., FlowJo, TreeStar Inc., Ashland, OR)

3. METHODS

3.1. Isolation and preparation of SVCs from mice

In this section, we describe the dissection of mouse adipose tissue and subsequent recovery of SVCs by collagenase digestion. We recommend using at least one entire perigonadal fat pad for flow cytometry analysis and FACS because regional differences in the distribution of leukocytes in visceral fat from mouse models have been noted (Cho et al., 2007). Experimental design should also account for the need for additional SVCs for compensation controls, which will be explained in detail in Section 3.3. We have found that minced tissue size, shaking intensity, and incubation time in collagenase buffer are critical parameters that should be optimized to maximize the yield of viable SVCs from visceral fat. Increasing surface area by finely mincing the tissue and frequent manual shaking during the digestion tends to increase SVC yield. Importantly, the duration of collagenase exposure should be empirically determined for each lot of collagenase. Increasing incubation times in collagenase buffer, especially longer than 1 h, significantly decrease SVC yield and increase cell death and should be avoided.

3.1.1 Isolation of adipose tissue

1. Euthanize mouse according to approved procedures and disinfect the skin with 70% ethanol.
2. Open the thoracic cavity to expose the heart. Perform a cardiac perfusion to remove blood from tissues. Using a 10 cc syringe and 25G needle, slowly perfuse the left ventricle with 10 ml of PBS. At the same time, puncture the right atria to allow blood and perfusate to escape the circulation.
3. Isolate the perigonadal adipose depot using sterile technique. Remove any visible gonadal tissue. When isolating mesenteric or inguinal adipose depots, care should be taken to remove lymph nodes.
4. Weigh the isolated fat pads and note the value. This weight will be used to normalize flow cytometry data.

5. Wash the adipose tissue with PBS to remove any contaminants, such as fur, and place tissue in a plastic weigh boat on ice.

3.1.2 Isolation of SVCs by collagenase digestion

1. Mince adipose tissue into small pieces with scissors (approx. 3–5 mm in size) in a weigh boat on ice.
2. Transfer minced tissues (≤~1 g) into 10-ml round-bottom tube containing 7 ml of ice-cold digestion buffer and keep on ice. For fat pads >1 g, mince the tissue and transfer into a 50-ml conical tube containing 10 ml of ice-cold digestion buffer. When tissue is added to buffer, mince further, and return the tube to ice until all samples have been harvested.
3. Add 1 ml (or 1.5 ml for >1 g) of 10× collagenase buffer. Adjust the final concentration of collagenase to 1 mg/ml by adding additional digestion buffer.
4. Incubate at 37 °C for 20–45 min with vigorous shaking using a test tube rocker. Higher cell yields can be achieved when the tubes are vigorously shaken by hand every 10 min. After 30 min, 10 μl of the digestion mixture should be examined microscopically. At this point, adipocytes should appear as large single cells; leukocytes and other stromal cells will be much smaller. If leukocytes are still attached to adipocytes, the digestion should continue; if not, proceed to the next step.
5. After digestion, add EDTA to a final concentration of 10 m*M* and incubate at 37 °C for an additional 5–10 min. This step is necessary to facilitate full dissociation of SVCs; EDTA exposure should be limited to avoid adversely effecting cell viability.
6. Prewet a 100-μm nylon filter with PBS and place onto a 50-ml conical tube. Using a transfer pipette, transfer the bottom layer of cell slurry onto the filter, followed by the adipocyte containing upper layer. This prevents larger adipocytes from clogging the filter before SVCs can pass freely through. Wash the filter twice gently by adding 10 ml of FACS buffer.
7. Centrifuge cell slurry at 500×*g* for 10 min at 4 °C to separate adipocytes and SVCs. After centrifugation, adipocytes form a white layer on top while SVCs form a red/white pellet on bottom of the tube.
8. Gently aspirate and discard adipocytes and supernatant. At this point, the SVC-containing pellet will be easy to disturb, so great care should be taken. If necessary, adipocytes (floating layer) can be collected using a transfer pipette prior to aspiration.
9. Manually disrupt pellet by flicking the tube and resuspend in 0.5 ml RBC lysis buffer. Incubate 5 min at room temperature with occasional gently shaking.
10. Neutralize RBC lysis by adding 5 ml of FACS buffer.
11. Centrifuge at 500×*g* for 10 min at 4 °C. Resuspend cell pellets in 3–5 ml FACS buffer and incubate on ice.

12. Mix 10 μl of each sample 1:1 with trypan blue solution. Count viable cells carefully using hemocytometer. The resulting cell number and total volume give an estimate of the number of SVCs per fat pad. Typical yields are 1–3×10^6 cells/g fat from lean mice, 2–5×10^6 cells/g fat from obese mice.

3.2. Staining SVCs for cell surface markers to identify ATMs

For multiparameter flow cytometry, the selection of antibodies and fluorochrome conjugates is a critical step (Baumgarth & Roederer, 2000; Maecker, Frey, Nomura, & Trotter, 2004). It has been our experience that ATMs from obese mouse fat have a significant amount of autofluorescence when excited by the blue laser (488 nm); thus, we try to avoid using FITC- or Alexa488-conjugated antibodies in our staining protocols for ATMs. The first step in selecting fluorochromes is to consider the flow cytometry or FACS instrument on which the samples will be analyzed. We recommend using instruments with at least a three-laser configuration (e.g., FACSCanto II equipped with 405 nm violet laser, 488 nm blue laser, and 640 nm red laser) as this will provide the greatest flexibility and allow for five to seven colors to be detected; however, if such an instrument is not available, two-laser configurations (e.g., 488/640 nm or 561/640 nm) are the next best option. When selecting fluorochrome conjugates, it is generally recommended to use those with the highest staining index (e.g., PE, PE-Cy5, APC) for rare cellular events, but care must be taken to minimize spectral overlap. Detailed discussions of these issues are presented elsewhere (Baumgarth & Roederer, 2000; Maecker et al., 2004). Once the antibodies and fluorochrome conjugates have been chosen, preliminary experiments should be performed to titer the antibody concentrations to maximize separation of positive and negative cell populations. Based on the antibody selection and titration data, SVCs can be stained with antibody cocktails (containing antibodies at predetermined concentrations in staining buffer) as described in the basic protocol below. For controls, additional SVCs are stained with each antibody individually (SS controls) and the antibody cocktail minus one antibody (FMO controls). Table 16.2 shows a summary of the control tubes needed in this antibody panel.

1. Transfer 1×10^6 SVCs from Section 3.1 into a 5-ml polystyrene round-bottom tube and resuspend in 100 μl of FACS buffer to obtain a final concentration 1×10^7 SVCs/ml. *Note*: When preparing cells for sorting, the total number of cells can be increased, but each reagent should be linearly increased as well.
2. Add 0.5–1 μg of Fc-block (anti-CD16/32) and incubate on ice for 10 min.
3. Repeat steps 1–2 for each control tube listed in Table 16.2. SVCs can be pooled from multiple mice for the SS and FMO controls. If cells are limiting, as few as 1×10^5 SVCs can be used for control tubes.
4. For each sample, prepare an antibody staining cocktail for ATMs as indicated in Table 16.1. Bring the cocktail to 100 μl/sample by adding FACS buffer.
5. Add 100 μl of antibody cocktail to each sample tube.
6. Flick tube to mix.
7. For SS and FMO controls, add the antibody or antibodies as indicated in Table 16.2.

8. Incubate all tubes for 30 min at 4 °C protected from light.
9. Wash cells with 2 ml FACS buffer and centrifuge 10 min at 500×*g* at 4 °C.
10. Carefully, aspirate supernatants.
11. Wash cells again with 2 ml FACS buffer and centrifuge 10 min at 500×*g* at 4 °C.
12. Carefully, aspirate supernatants.
13. Optional (for flow cytometry analysis only): stain the samples and appropriate controls with viability dye (fixable live/dead stain). Dilute dye (1:500–1:1000) in 200 μl PBS/sample. Incubate the cells in dye solution at room temperature for 30 min. Repeat steps 9–12 to wash cells. Proceed to step 14.
14. For flow cytometry analysis, fix cells by adding 200 μl of 0.1% PFA. Cells can be stored in PFA at 4 °C in the dark until data acquisition. Prolonged exposure in PFA may increase autofluorescence; therefore, it is advisable to remove PFA after 1 h and replace with 200–300 μl of FACS buffer if cells will not be analyzed within 24 h.
15. For sorting of viable cells by FACS, suspend cells in FACS buffer to obtain a final concentration suitable for sorting (generally 1–10×10^7/ml). Add DAPI (0.2 μg/ml) to each sample and appropriate controls to allow for live/dead cell discrimination. Protect tubes from light and place on ice for transport to the FACS instrument.

3.3. Flow cytometry analysis

3.3.1 Compensation procedures—As noted above, care should be taken to minimize emission spectra overlap when selecting fluorochrome-conjugated antibodies for ATMs. However, it is difficult to completely eliminate spectral overlap in multicolor staining. Therefore, it is necessary to perform a compensation procedure during each experiment before acquiring data from the samples of interest. Each instrument has a different compensation procedure, with some software packages generating a compensation matrix automatically from SS controls. Therefore, we recommend consulting the manual and following the manufacturer's recommendations for your instrument. When compensation is correctly applied, the median fluorescence intensities (MFIs) of the positive and negate populations of the individual SS controls are aligned in all neighboring channels. It is important to use SS controls prepared from SVCs (Section 3.2, Step 3) because these cells will have the same properties as the samples of interest. Here, we provide a brief guide for getting started.

1. Using an unstained control sample, adjust side scatter and forward scatter so that the cell populations of interest are on scale.
2. Using the unstained control sample, adjust the photomultiplier tube (PMT) gain for each fluorochrome detector so that the peak MFI of the unstained cells on a histogram is within 10^1–10^2 on a log scale.
3. Acquire all SS compensation controls. If necessary, adjust the PMT gains for each detector so that the positive (stained) population can be discriminated from the

negative (unstained) cells. We acquire and save 10,000–30,000 events for each SS control.

4. Calculate compensation values across all included detector according to instructions for your instrument. Importantly, apply compensation values to all SS controls, FMO controls, and samples of interest.

3.3.2 Data acquisition

1. Using the same instrument settings, acquire all FMO controls individually. FMO controls contain all the antibodies in the staining cocktail, but one antibody is replaced with an isotype control. For example, FMO-APC contains the APC-isotype antibody rather than APC-CD301 (Table 16.2). This is necessary for discriminating positive from negative populations during subsequent gating of the samples of interest. Acquire and save 10,000–30,000 events.
2. Acquire and save 10,000–50,000 events from the samples of interest. To generate statistically sound data for frequency determination, a sufficient number of events need to be obtained; this may require recording more than 50,000 events per sample.

3.3.3 Gating strategy to identify and characterize ATMs—Figure 16.1 depicts our general strategy for identifying M1 ($CD11c^+$) and M2 ($CD301^+$) ATMs for quantification and purification by FACS. Cell aggregates, dead cells ($DAPI^+$), and cellular debris are first excluded (Fig. 16.1A). This step greatly reduces, but does not eliminate, autofluorescence in SVC preparations. Next, adipose tissue leukocytes ($CD45^+$) are selected. ATMs from both lean and obese mice coexpress F4/80 and CD11b (Fig. 16.1A). However, $F4/80^{mid}$ cells (gate 2) contain eosinophils ($Siglec\text{-}F^+$) and neutrophils ($GR1^+$; Fig. 16.1B); therefore, care should be taken to gate ATMs as $F4/80^{high}CD11b^{high}$ (gate 1) to minimize contamination with these cell types. Finally, viable $CD45^+F4/80^{high}CD11b^{high}$ ATMs are analyzed for surface expression of CD11c (M1 marker) and CD301 (M2 marker), which identify discrete M1 and M2 ATM subsets in both lean and obese mice (Figs. 16.1A and 16.3B).

3.4. FACS procedure to purify ATM populations

1. Prior to FACS, decide the number of desired target cells to be collected. Cells should be sorted directly into the appropriate media (e.g., cell culture media, lysis buffer) for the downstream application. Prepare enough collection tubes for the cells of interest.
2. Prepare SVCs from adipose tissue as described in Section 3.1.
3. Stain SVCs with antibody cocktail as described in Section 3.2. Cells should be transported to the FACS facility on ice and protected from light.
4. Set up the FACS instrument according to the manufacturer’s directions.
5. Run SS and FMO controls to setup compensation matrix and sorting gates.
6. Optimize the FACS instrument for sorting according to the manufacturer’s protocol and adjust speed of sample acquisition to optimize purity of the sample.

7. Vortex the source cells and run a test sort.
8. Continue sorting cells until the desired cell number is achieved or the source cells are exhausted.

3.5. Using magnetic beads and positive selection to enrich for CD11b$^+$ SVCs

In this section, we describe the enrichment of CD11b$^+$ leukocytes from SVCs using magnetic beads and positive selection (Fig. 16.2). The majority of the cells in this pool will be ATMs but contamination with neutrophils and eosinophils will be likely. Downstream *in vitro* studies can further enrich for ATMs by adhesion to plastic.

1. In a 5-ml polystyrene round-bottom tube, suspend 10^7 SVCs in 90 μl of MACS buffer.
2. Add 10 μl of CD11b microbeads.
3. Mix well by flicking tube or very gently vortexing. Incubate for 15 min at 4 °C.
4. Wash cells by adding 1 ml of MACS buffer and centrifuge at 500×*g* for 10 min at 4 °C. Discard supernatant.
5. Resuspend cell pellet in 500 μl of MACS buffer.
6. Place MS separation column in magnetic field of MACS separator. Equilibrate column by adding 500 μl of MACS buffer.
7. Apply cell suspension to center of column. Collect unlabeled (CD11b$^-$) cells that pass through the column in a 5-ml tube.
8. Wash column three times with 500 μl of MACS buffer, allowing the column to empty with each wash. Do not allow the column to dry. Collected flow through (CD11b$^-$ fraction) can be assayed by flow cytometry if necessary.
9. Remove MS column from the magnetic field. Place in a 5-ml tube.
10. Add 1 ml of MACS buffer to the column. Using the provided plunger, expel the CD11b$^+$ fraction into the tube.
11. Centrifuge cell fractions at 500×*g* for 5 min at 4 °C. Discard supernatant, and resuspend cell pellets in 300 μl of FACS buffer and stain for cell surface antigens or further analysis.

3.6. Data analysis

1. Analyze flow cytometry data with FlowJo software.
2. Determination the frequency of ATMs and ATM subsets as a percentage of total viable cells (Fig. 16.3C and D).
3. Based on the % of SVCs, ATM content can also be reported as absolute cell number (%ATMs×total SVC isolated), or normalized to adipose tissue mass (%ATMs×total SVCs/fat pad weight (g) (Fig. 16.3C and D).
4. Perform statistical analysis.

4. DISCUSSION

The approach presented here has evolved over the years in our laboratory and has undergone a variety of refinements to best assess ATM in a variety of mouse models (Lumeng et al., 2011; Morris et al., 2013; Morris, Oatmen, Wang, DelProposto, & Lumeng, 2012; Singer et al., 2013; Westcott et al., 2009). Such techniques provide the most reliable quantitation of ATM content and assess the balance between proinflammatory M1 ($CD11c^+$) ATMs and anti-inflammatory M2 ($CD301^+$) ATMs in lean and obese mice. This FACS strategy has also been successfully used to purify ATMs for gene expression analysis and functional assays (Morris et al., 2013; Singer et al., 2013). While similar principles may apply for analysis of human ATMs, Hagman et al. (2012) provide an excellent strategy specifically tailored to human samples.

In our experience, the most critical steps in the protocol are efficient digestion of adipose tissue by collagenase and proper staining and compensation of SVCs. We have found that gentle extraction techniques optimized for study of adipocyte function do not efficiently remove ATMs from the floating adipocytes during the differential centrifugation step. The upper layer can be contaminated with lipid-laden ATMs, which are buoyant, with ATMs that remain attached to adipocytes without vigorous digestion. Attempts to combine analysis of SVCs with adipocyte function have been challenging, as digestion strategies need to be optimized for each application.

Our strategy to identify ATMs is based on initial observations that macrophages in adipose tissues coexpress F4/80 and CD11b (Weisberg et al., 2003). Since then, it has been established that $F4/80^{mid}$ cells in fat from mice can also contain eosinophils (Siglec-F^+) and neutrophils (GR-1^+), although they are minor populations compared to ATMs (Fig. 16.1B) (Talukdar et al., 2012; Wu et al., 2011). In our staining protocols, ATMs are best identified as $F4/80^{high}CD11b^{high}$ (Fig. 16.1A; gate 1).

How to appropriately normalize ATM data from flow cytometry continues to be a challenge in fat depots that massively expand in size due to adipocyte hypertrophy. In our example data (Fig. 16.3C), SVCs from lean mice contains 9% ATMs and high-fat diet (HFD) exposure for 12 weeks increased ATM content to 15% (1.6-fold increase). However, the absolute and relative (cells/g fat) number of ATMs increased approximately threefold (Fig. 16.3C). In agreement with previous reports (Li et al., 2010; Lumeng et al., 2008), HFD-induced obesity also altered the distribution of M1 and M2 ATM subtypes in visceral fat (Fig. 16.3B and D). ATM content and the frequencies of M1 versus M2 ATMs obtained here are comparable to other studies (Weisberg et al., 2003; Xu et al., 2003), indicating that our flow cytometry method provides a simple and reproducible method for assessing the content and heterogeneity of ATMs in mice models.

One obstacle that limits flow analysis is the significant cellular autofluorescence of adipose SVCs and ATMs. Especially in obese mice, ATMs are embedded in the autofluorescent $SSC^{hi}FSC^{hi}$ populations of cells that are prominent in the SVC. Analyses should not exclude these cells as this will significantly underreport ATM content. Gating to exclude dead cells, debris, and cell aggregates can improve results, but a significant amount of autofluorescence

from ATMs remains likely due to high lipid content. In our experience, these limitations almost completely exclude the routine use of FITC- and Alexa488-conjugated monoclonal antibodies for macrophages in staining cocktails. This limitation can be partially overcome by using flow cytometers with yellow–green (561 nm) lasers versus the conventional blue (488 nm) laser configuration (Morris, D.L., unpublished observation).

For those starting such a procedure for the first time, consultation with flow cytometry experts is highly recommended prior to performing experiments. Over time this protocol has been shown to give highly reproducible results and we feel is a good starting point for those looking to examine ATMs in their mouse models. Additional refinements of the protocol should continue by all groups in this field as it continues in our laboratory.

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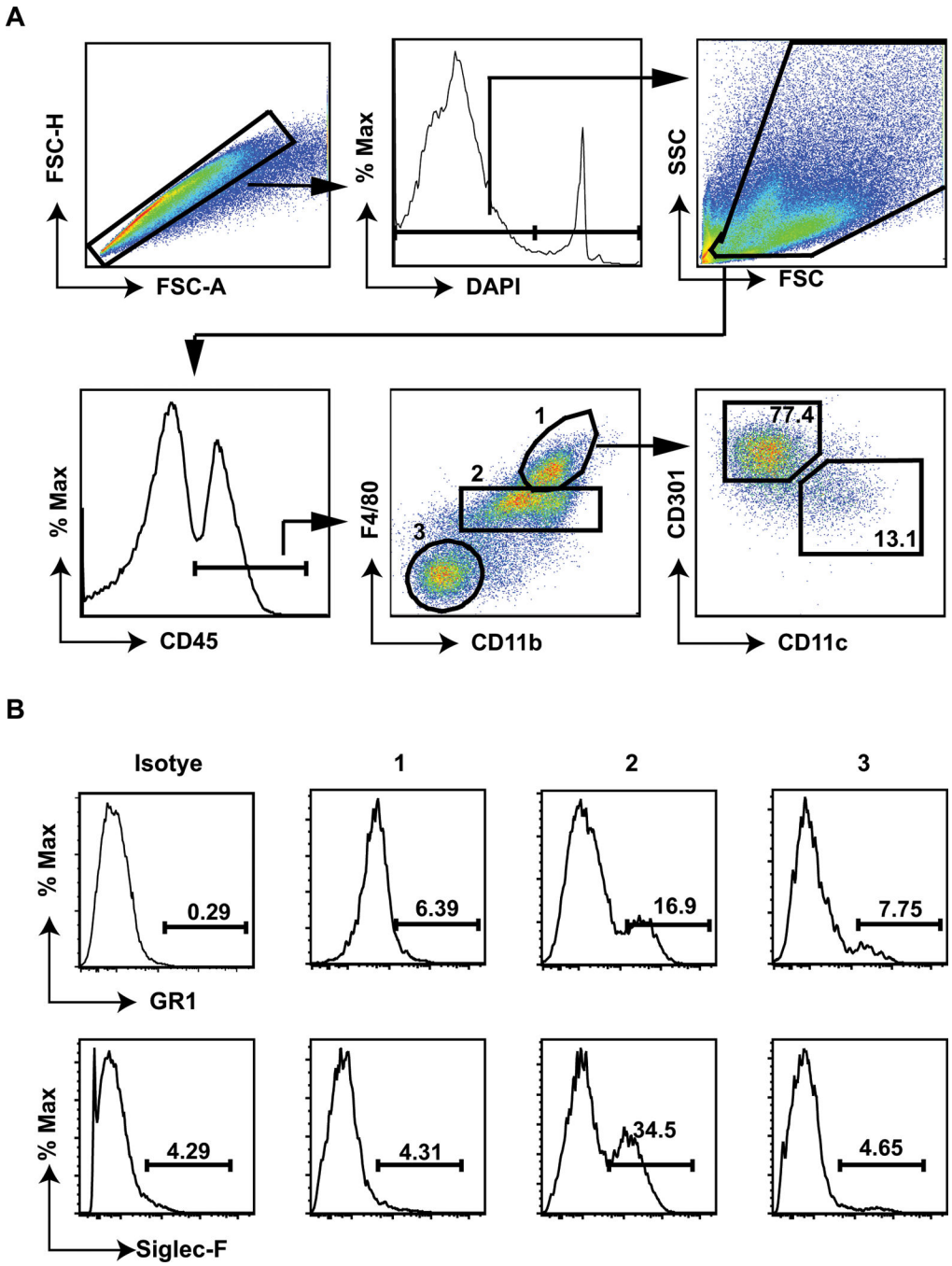

Figure 16.1.

Gating strategy to identify and characterize ATMs. (A) Gating strategy for the identification of M1 ($CD11c^{+}$) and M2 ($CD301^{+}$) ATMs according to the flow cytometry protocol described herein. (B) Histograms of Gr-1 and Siglec-F expression on $CD11b^{high}F4/80^{high}$ (gate 1), $CD11b^{mid/high}F4/80^{dim}$ (gate 2), and $CD11b^{-}F4/80^{-}$ (gate 3) SVCs demonstrates that the $CD11b^{mid/high}F4/80^{dim}$ population (gate 2) can be contaminated with neutrophils (Gr-1) and eosinophils (Siglec-F).

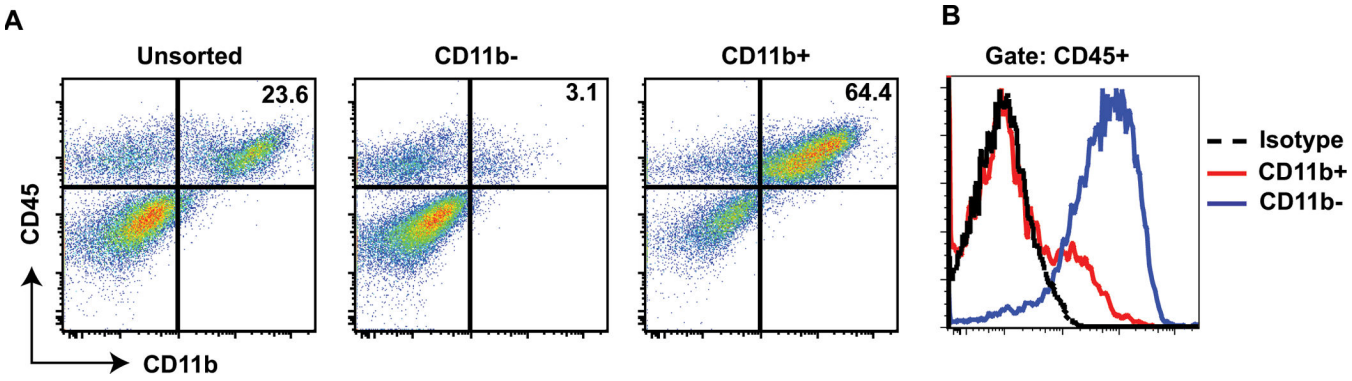

Figure 16.2.

Enrichment of CD11b$^+$ SVCs using magnetic beads. SVCs were isolated as described in Section 3.1 and CD11b$^+$ cells were positively selected using Miltenyi microbeads as described in Section 3.5. Unsorted and sorted cells were analyzed by flow cytometry. (A) Scatterplots showing CD45 versus CD11b expression on unsorted (left), CD11b-depleted (CD11b$^-$; middle), and CD11b-enriched (CD11b$^+$; right) SVCs. The percentage of total cells for the CD45$^+$CD11b$^+$ population is indicated. (B) Histogram showing enrichment efficiency of CD11b$^+$ SVCs following positive selection.

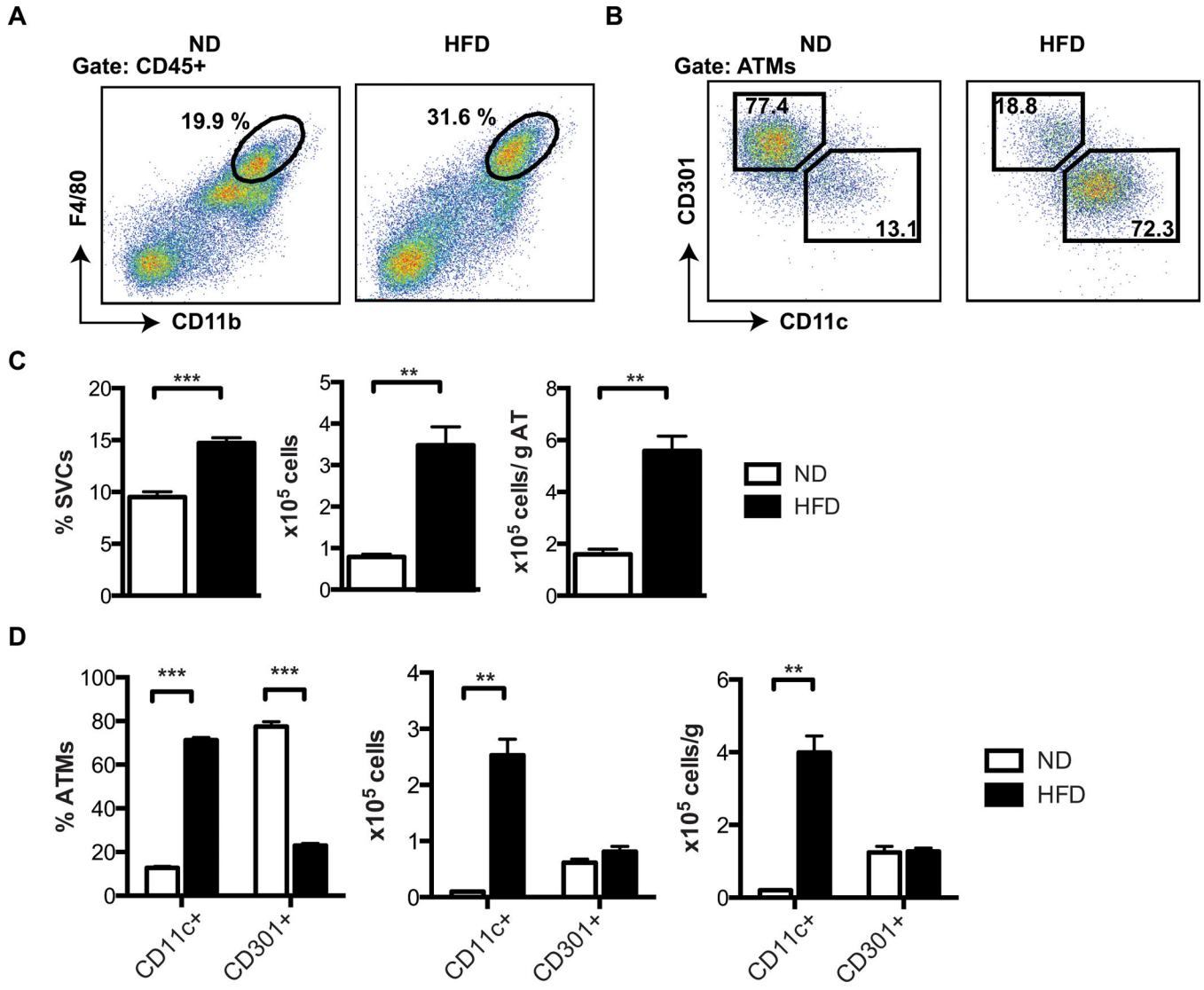

Figure 16.3.

High-fat diet increases ATM content and alters the distribution of M1 and M2 ATMs in visceral fat. C57BL/6J mice were fed a normal diet (ND) or high-fat diet (HFD; 60 kcal from fat) for 12 weeks before stromal vascular cells (SVCs) were isolated from epididymal adipose tissue and examined by flow cytometry as described in these protocols. (A) Representative scatterplots of SVCs from ND (left) and HFD (right) mice stained with CD11b and F4/80. Cells are gated on viable $CD45^+$ SVCs as shown in Fig. 16.1A. (B) Representative scatterplots showing gates for M1 ($CD11c^+CD301^-$) and M2 ($CD11c^-CD301^+$) ATMs. (C) ATM content in epididymal adipose tissue shown as percentage of total SVCs, absolute number per fat pad, and normalized to visceral adipose tissue mass. (D) Distribution of ATM subsets in visceral fat from lean and obese mice shown as percentage of total ATMs, absolute number per fat pad, and normalized to adipose tissue mass. $^{**}P<0.01$, $^{***}<0.001$ versus ND.

Table 16.1

Basic antibody cocktail for ATM staining

Antibody	Clone	Supplier	Titrated concentration (μg/10^6 cells)
F4/80 PE	BM8	eBioscience	0.2
CD11b APC eFluor 780	M1/70	eBioscience	0.16
CD11c PE-Cy7	N418	eBioscience	0.2
CD45.2 PerCP-Cy5.5	104	eBioscience	0.2
CD301 Alexa Fluor 647	ER-MP23	AbD Serotec	0.2

Table 16.2

Single stained (SS) and fluorescence minus one (FMO) controls for ATM staining

	Pacific Blue	PE	PE-Cy7	PerCP5.5	APC	APC-Cy7
Unstained						
SS-Pacific	Live/dead					
SS-PE		F4/80				
SS-PECy7			CD11c			
SS-PerCP5.5				CD45		
SS-APC					CD301	
SS-APCCy7						CD11b
FMO Pacific	Isotype	F4/80	CD11c	CD45	CD301	CD11b
FMO PE	Live/dead	Isotype	CD11c	CD45	CD301	CD11b
FMO PE-Cy7	Live/dead	F4/80	Isotype	CD45	CD301	CD11b
FMO PerCP5	Live/dead	F4/80	CD11c	Isotype	CD301	CD11b
FMO-APC	Live/dead	F4/80	CD11c	CD45	Isotype	CD11b
FMO-APC-Cy7	Live/dead	F4/80	CD11c	CD45	CD301	Isotype

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